Serum Concentrations of Lipopolysaccharide-Binding Protein as a Biochemical Marker to Differentiate Microbial Etiology in Patients with Community-Acquired Pneumonia, Mar Masía,1,* Félix Gutierrez,1 Belén Llorca,2 Juan Carlos Navarro,2 Carlos Mirete,2 Sergio Padilla,1 Ildefonso Hernández,3 and Emilio Flores2 (1 Infectious Diseases Unit, Internal Medicine Department, and 2 Clinical Chemistry Service, Hospital General Universitario de Elche, Alicante, Spain; 3 Public Health Department, Universidad Miguel Hernández, Alicante, Spain; * address correspondence to this author at: Unidad de Enfermedades Infectiosas, Hospital General Universitario de Elche, Camí de l’Almazara, 11, 03203 Elche, Alicante, Spain; fax 34-96 6679156, e-mail marmasia@ya.com)

Community-acquired pneumonia (CAP) is a major cause of morbidity and mortality. However, identification of the infecting organism is achievable in only 40–50% of cases, and results are not usually available when the diagnosis of pneumonia is first established (1, 2). Therefore, initial therapy is typically empirical, usually aimed at the standard bacteria, most commonly Streptococcus pneumoniae, and “atypical” pathogens (e.g., Mycoplasma pneumoniae, Legionella species, Chlamydiophila species, and Coxiella burnetii) that have long been considered the most frequent causes of CAP. Given their effectiveness against S. pneumoniae as well as atypical organisms, macrolide antibiotics have been widely used as initial monotherapy in many cases of CAP. The selection of empirical therapy has become complicated by the emergence of drug-resistant S. pneumoniae, including a growing number of strains resistant to macrolides (1–3). In light of these uncertainties, clinicians may be inclined to prescribe broad-spectrum antimicrobial coverage for many patients with CAP, a practice that could lead to increasing bacterial resistance over the next few years (3).

A patient-specific therapy for CAP that relies on antibiotics with a focused spectrum of activity could improve the care of the individual patient and prevent antibiotic abuse, reducing the risk of microorganisms becoming drug resistant. Therefore, the development of biomarkers to help clinicians predict the microbial etiology of CAP could be useful for selecting patient populations in which this strategy may be appropriate and effective. In fact, a therapeutic strategy based on the concentration of procalcitonin (PCT), a marker of bacterial infection, has recently demonstrated usefulness in reducing antibiotic overuse in lower respiratory tract infections (4).

Lipopolysaccharide-binding protein (LBP) is the principal plasma protein responsible for transporting endotoxin molecules to immune effector cells bearing CD14 on their surface (5). High serum LBP concentrations have been found in sepsis caused by gram-negative and -positive bacteria (6, 7). To the best of our knowledge, serum LBP concentrations have not been evaluated in CAP. Because LBP appears to be a marker for bacterial infection, it might be useful in distinguishing between pneumonia caused by standard bacteria, which could usually be treated with β-lactam antibiotics, and pneumonia caused by atypical microorganisms, which is best treated with a macrolide.

The aims of the present study were to measure the serum concentrations of LBP in patients with CAP and to explore their usefulness in predicting microbial etiology.

A prospective population-based study of CAP was conducted over a 24-month period (October 1999 through October 2001) at Hospital Universitario de Elche, a 430-bed teaching hospital in Spain. Eligible patients were all adults (>15 years) with signs and symptoms of CAP. CAP was defined as an acute illness associated with at least one of the following signs or symptoms: fever, new cough with or without sputum production, pleuritic chest pain, dyspnea, or altered breath sound on auscultation; in addition, radiographs of the chest were examined for an opacity compatible with the presence of acute pneumonia. The study was approved by the local Ethics Committee, and informed consent was obtained from all participants. All patients were evaluated clinically and roentgenographically at the hospital Emergency Room, where the diagnostic criteria of pneumonia were applied; those patients with a provisional diagnosis of CAP were seen by a study investigator to confirm diagnosis. The patients were then admitted or managed as outpatients. An extensive noninvasive microbiological investigation was performed in all patients. The laboratory work-up and the criteria used to classify the pneumonia as being of known etiology have been described in detail elsewhere (8).

During the first 12-month period, from October 15, 1999, to October 15, 2000, all patients included in the study had a blood sample collected, within the first 24 h after fulfilling the pneumonia criteria, to measure LBP concentrations. Samples were centrifuged, decanted, aliquoted, and stored frozen at −80 °C until analyzed in May 2003. In addition to LBP, other serum biomarkers, such as C-reactive protein (CRP) and PCT, were also measured.

LBP was measured by a commercially available assay (IMMULITE LBP; DPC) according to the instructions of the manufacturer (9); the limit of detection for this assay is 0.2 mg/L, and the absolute range in healthy individuals is 2.0–15.2 mg/L. According to the DPC protocol for LBP, 10 μL of serum is prediluted in 1000 μL of the LLBZ4 LBP sample diluent (dilution, 1:101). The sample is then pipetted into a cuvette test containing a bead coated with monoclonal murine anti-LBP antibody and incubated, with intermittent agitation, with an alkaline phosphatase-labeled polyclonal rabbit anti-LBP antibody and washed. Sustained light emission is detected after injection of a phosphate ester of adamantyl dioctetane (cheluminescent substrate) and is proportional to serum LBP concentrations. The intra- and interassay CVs for the assay used were <11%. The test did not give indeterminate results.

We compared serum LBP concentrations in patients with pneumonia caused by atypical pathogens with those found in the rest of the patients with pneumonia; we also compared them specifically with the concentrations in patients diagnosed as having bacterial pneumonia, viral pneumonia, or pneumonia of unknown etiology. Statisti-
cal analysis was performed with SPSS, Ver. 8. Data were compared by use of the Mann–Whitney U-test. The results are presented as median values with 25–75 interquartile ranges. A P value <0.05 was considered significant. ROC curves were used to describe the sensitivity and specificity of classification for each LBP cutoff point. The sensitivity and specificity of the LBP cutoff point to differentiate atypical from nonatypical pneumonia in patients under 45 and 35 years of age were also determined with ROC curves.

Of 251 patients evaluated from October 15, 1999, to October 15, 2000, eleven were subsequently found not to have CAP, leaving 240 patients in the study cohort. The mean age was 59 years (range, 15–93), and 62.5% were male. In 115 (48%) patients there was one or more underlying disease, mostly diabetes mellitus (n = 55) or chronic obstructive pulmonary disease (n = 51). One hundred ninety-one (79.6%) patients were admitted to hospital, 4 (1.7%) of whom required admission to the intensive care unit. The remaining 49 (20.4%) patients were managed as outpatients. The main symptoms described by the patients were the following: cough in 202 (84.2%); dyspnea in 151 (62.9%); purulent sputum production in 141 (58.8%); pleuritic chest pain in 132 (55%); and chills in 116 (48.3%).

Of the 240 patients studied, 104 (43.3%) had an axillary body temperature higher than 38°C at the time of diagnosis. The causative pathogen was found in 105 (43.7%) patients (33 bacterial, 44 atypical, 17 viral, 11 mixed). In 191 (64.2%) patients, the pneumonia resolved without complications, whereas 49 (20.4%) developed one or more of the following complications: absence of response to treatment (n = 31; 12.9%); atelectasis (n = 23; 9.6%); septic shock (n = 6; 2.3%); mechanical ventilation requirement (n = 4; 1.7%); empiema (n = 2; 0.8%); and readmission (n = 8; 3.3%). Seventeen (7.1%) patients died.

Serum LBP was measured in 196 (81.7%) patients. For the remaining patients, the test was not performed because a serum sample was not available or the volume was insufficient. There were no differences in age, sex, comorbidity, or Fine’s score between patients for whom LBP was measured and those for whom it was not (data not shown). CRP was also measured in 196 patients and PCT in 185. The median CRP concentration was significantly lower in patients with pneumonia caused by atypical organisms (3.7 mg/L; range, 3.7–150 mg/L) than in the rest of the patients with pneumonia (13.7 mg/L; range, 3.7–454 mg/L; P <0.001) or in those with bacterial pneumonia (26.4 mg/L; range, 3.7–343 mg/L; P = 0.002) and viral pneumonia (11.4 mg/L; range, 3.7–211 mg/L; P <0.001). We observed no differences in the serum PCT concentrations among major etiologic groups (data not shown).

The median LBP values according to the etiology of the pneumonia are shown in Table 1. The median LBP was 10 mg/L in patients with pneumonia caused by atypical pathogens vs 22.2 mg/L in the rest of the patients (P <0.001). Serum LBP concentrations were significantly lower in patients with pneumonia caused by atypical

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### Table 1. Serum LBP concentrations in patients with CAP according to etiology.

<table>
<thead>
<tr>
<th>Etiology</th>
<th>No. of patients</th>
</tr>
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<tbody>
<tr>
<td>Atypical</td>
<td>39</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>16</td>
</tr>
<tr>
<td>Legionella pneumophila</td>
<td>12</td>
</tr>
<tr>
<td>Chlamydia psittaci</td>
<td>5</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>3</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>3</td>
</tr>
<tr>
<td>Bacterial</td>
<td>24</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>9</td>
</tr>
<tr>
<td>Hemophilus influenzae</td>
<td>6</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>4</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
</tr>
<tr>
<td>Viral</td>
<td>16</td>
</tr>
<tr>
<td>Influenza/parainfluenza virus</td>
<td>12</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
</tr>
<tr>
<td>Mixed</td>
<td>11</td>
</tr>
<tr>
<td>Bacterial + atypical</td>
<td>3</td>
</tr>
<tr>
<td>Atypical + viral</td>
<td>2</td>
</tr>
<tr>
<td>Two atypical microorganisms</td>
<td>5</td>
</tr>
<tr>
<td>Two viruses</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>106</td>
</tr>
<tr>
<td>Total</td>
<td>196</td>
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</table>

<table>
<thead>
<tr>
<th>LBP, mg/L</th>
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<tbody>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Interquartile range</td>
</tr>
<tr>
<td>Minimum</td>
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<tr>
<td>Maximum</td>
</tr>
</tbody>
</table>

- **Atypical**: 10.00 (7.00–14.60) mg/L, 3.10–75.40 mg/L
- **Mycoplasma pneumoniae**: 10.40 (6.10–14.13) mg/L, 3.90–52.30 mg/L
- **Legionella pneumophila**: 9.80 (7.05–15.68) mg/L, 3.10–23.30 mg/L
- **Chlamydia psittaci**: 14.50 (9.20–62.35) mg/L, 9.20–75.40 mg/L
- **Chlamydia pneumoniae**: 5.70 (5.60–10.00) mg/L, 5.60–10.0 mg/L
- **Coxiella burnetii**: 7.80 (7.40–16.90) mg/L, 7.40–16.90 mg/L
- **Bacterial**: 21.80 (11.95–39.83) mg/L, 4.20–78.20 mg/L
- **Streptococcus pneumoniae**: 20.40 (11.25–29.80) mg/L, 4.20–78.20 mg/L
- **Hemophilus influenzae**: 21.10 (10.65–35.58) mg/L, 7.20–41.80 mg/L
- **Pseudomonas aeruginosa**: 23.95 (20.18–41.53) mg/L, 20.0–46.30 mg/L
- **Other**: 41.80 (10.00–43.65) mg/L, 8.20–44.40 mg/L
- **Viral**: 19.90 (12.75–34.70) mg/L, 4.60–80.00 mg/L
- **Influenza/parainfluenza virus**: 23.20 (14.75–36.83) mg/L, 4.60–80.00 mg/L
- **Other**: 16.45 (11.70–20.60) mg/L, 11.50–20.60 mg/L
- **Mixed**: 9.60 (6.90–27.80) mg/L, 6.50–101.00 mg/L
- **Bacterial + atypical**: 27.80 (6.70–101.00) mg/L, 6.70–101.00 mg/L
- **Atypical + viral**: 7.20 (6.70–15.35) mg/L, 6.50–22.90 mg/L
- **Two atypical microorganisms**: 7.20 (6.70–15.35) mg/L, 6.50–22.90 mg/L
- **Two viruses**: 9.60 (9.60–27.80) mg/L, 6.50–101.00 mg/L
- **Unknown**: 14.75 (9.85–31.48) mg/L, 2.20–108.0 mg/L
- **Total**: 14.40 (9.20–29.58) mg/L, 2.20–108.0 mg/L

- **Staphylococcus aureus**: 2 cases; other gram-negative bacilli: 3 cases.
- **Respiratory syncytial virus**: 3 cases; adenovirus: 1 case.
organisms than in those with bacterial or viral pneumonia ($P < 0.001$ and $P = 0.003$, respectively).

In the ROC curves, a cutoff of 14 mg/L for LBP showed the best discriminatory power. Seventy-two percent of the patients with pneumonia caused by atypical organisms had concentrations below this cutoff, compared with 29.2% of those with bacterial pneumonia and 25% of patients with viral pneumonia. When we included in the analysis leukocyte counts, we found that a LBP value $<14$ mg/L combined with an absolute neutrophil count $<8.5 \times 10^{12}$/L differentiated between etiologic groups better than LBP alone. We found that 48.7% of patients with pneumonia caused by atypical organisms had both LBP $<14$ mg/L and a neutrophil count $<8.5 \times 10^{12}$/L, whereas only 4.2% of patients with bacterial pneumonia had values below these cutoffs.

Because LBP concentrations varied with age, we stratified accuracy according to age. When patients younger than 45 years were selected, a LBP cutoff of $<7.9$ mg/L captured 13 of the 22 patients with pneumonia caused by atypical organisms (59% sensitivity) and 3 of the 30 patients with pneumonia of any other etiology (90% specificity). In the group of patients younger than 35 years, a LBP cutoff of $<7.9$ mg/L captured 10 of the 17 patients with pneumonia caused by atypical organisms (59% sensitivity) and only 1 of the 18 patients with pneumonia of any other etiology (94% specificity).

Our findings indicate an association between serum LBP concentrations and microbial etiology in CAP. Pneumonia caused by atypical organisms was associated with lower LBP values than were bacterial pneumonia, viral pneumonia, and pneumonia of unknown origin. Because LBP has been mainly linked with bacterial sepsis (6), it was not surprising to find higher serum concentrations in patients with bacterial pneumonia than in those with pneumonia caused by atypical organisms. Although during acute bacterial infections a release of several cytokines leading to activation of the innate immune system and LBP synthesis has been demonstrated (6, 7, 10), no such a cascade has been described with atypical organisms. However, the increased LBP found in viral pneumonia was somewhat unexpected and should be interpreted with caution because, despite the extensive microbiological work-up carried out, an associated bacterial co-infection can not be definitively ruled out in some of these cases.

LBP is constitutively synthesized in hepatocytes after induction by interleukin-1 (IL-1) and IL-6 during the acute-phase response. In infections caused by gram-negative microorganisms, bacterial endotoxin or lipopolysaccharide (LPS) plays a central role in the pathophysiology of severe sepsis and septic shock. LBP recognizes LPS, and at low concentrations, it activates and amplifies the inflammatory host response by transporting LPS to effector cells of the immune system bearing CD14 on their surfaces, such as monocytes, macrophages, and neutrophils (6). These effector cells release proinflammatory mediators, including IL-1$\beta$, IL-8, IL-12, and tumor necrosis factor-$\alpha$, leading to the systemic inflammatory response syndrome. Interestingly, Zweigner et al. (10) showed that at high concentrations, LBP binding to monocytes and their subsequent activation, thereby inhibiting the proinflammatory activity of LPS.

The acute-phase increase in LBP concentrations may therefore represent an important part of the antimicrobial defense system of the host during bacterial infection (10). Low LBP concentrations have been associated with worse outcome in patients with severe sepsis (6).

Whereas there was a clear relationship between LBP concentrations and CAP etiology, both high and low LBP concentrations were found in all major groups, suggesting that in addition to the microbial agent, other factors explain the increase in the serum concentration of this protein. LBP has been described as a marker of inflammation, increasing not only in systemic inflammatory response and multiple organ dysfunction syndromes associated with bacterial infection, but also in nonspecific inflammatory syndromes such as hemorrhagic colitis or hemolytic uremic syndrome (11).

A cutoff of $<14$ mg/L was the most meaningful LBP value to differentiate pneumonia caused by atypical organisms from pneumonia of other etiology. When we considered LBP and neutrophil count together, serum LBP concentrations $<14$ mg/L and a neutrophil count $<8.5 \times 10^{12}$/L captured nearly one-half of the patients with pneumonia caused by atypical organisms and only 4% of patients with bacterial pneumonia. The accuracy of LBP was better in younger populations. Indeed, in patients under 35 years of age, serum LBP had a sensitivity of 59% and a specificity of 94% for diagnosing pneumonia caused by atypical organisms, indicating that one subset of patients could be treated confidently with a macrolide alone. Although it has limitations because of false-positive and -negative values, serum LBP may eventually be an additional diagnostic tool for diagnosing CAP when used in combination with other clinical or analytical indices.

The results of this study also support the high incidence of CAP caused by atypical pathogens reported by other investigators (12–14). In our cohort, evidence of acute infection with an atypical organism was found in 20% of patients with CAP and in $>40$% of the cases with an etiologic diagnosis. The frequency of pneumonia caused by atypical pathogens was higher than that of bacterial pneumonia. The inclusion of both ambulatory and hospitalized patients, the comprehensive serologic search for atypical pathogens, and the use of strict criteria to diagnose bacterial pneumonia by traditional microbiological procedures may have accounted for this etiologic distribution.

In summary, low serum LBP concentrations are associated with pneumonia caused by atypical organisms. LBP could aid in the initial management of CAP, helping clinicians to select patients in whom a narrow spectrum antibiotic therapy could be administered. Further studies will be required to confirm our results and to establish the clinical impact of LBP and its effectiveness in clinical practice.
Simultaneous Genotyping of Nine Polymorphisms in Xenobiotic-Metabolizing Enzymes by Multiplex PCR Amplification and Single Base Extension, Ad M. Knuutinen,1 Hans B. Ketelslegers,1 Ralph W.H. Gottschalk,1 Rob G.J.H. Janssen,2 Aimee D.C. Paulussen,2 Hubert J.M. Smeets,2 Roger W.L. Godschalk,1 Frederik J. Van Schooten,1 Jos C.S. Kleinjans,1 and Joost H.M. Van Delft*1*

(Departments of 1Health Risk Analysis and Toxicology and 2Population Genetics, Genomics and Bioinformatics, University of Maastricht, Maastricht, The Netherlands; * address correspondence to this author at: Department of Health Risk Analysis and Toxicology, University of Maastricht, PO Box 616, 6200 MD, Maastricht, The Netherlands; fax 31-43-3884146, e-mail j.vandelft@grat.unimaas.nl)

Studies have reported a large interindividual variation in susceptibility to health effects caused by exposure to xenobiotic compounds such as drugs or chemical carcinogens. There is evidence that this can be partly explained by the existence of genetic polymorphisms in metabolic enzymes, such as cytochrome P450 (CYP450), N-acetyltransferases (NATs), and glutathione S-transferases (GSTM, GSTT, GSTP) [see, e.g., Refs. (1–4)]. However, studies investigating associations between genetic polymorphisms and disease have reported conflicting results, probably caused by insufficient statistical power (5). Moreover, the majority of these studies focused on single polymorphisms. Regarding the number of genes implicated in the metabolism of xenobiotics and the large number of polymorphisms present in the human genome (6), these approaches fail to fully determine the role of genetic variation in an individual’s susceptibility to xenobiotic exposures. Such observations underline the need for methodologies that allow for high-throughput, low-cost genotyping of multiple polymorphisms in large populations (7, 8). In this study we describe the development, validation, and application of a cost-effective and rapid method for simultaneous genotyping of nine polymorphisms in five key enzymes involved in metabolism of xenobiotics: CYP1A2, GSTM1, GSTP1, GSTT1, and NAT2.

The fragments containing the nine single-nucleotide polymorphisms (SNPs) were amplified in one sevenplex and one duplex PCR reaction (Table 1). Primers were obtained from Qiagen. For the sevenplex PCR, a 50-µL reaction mixture was prepared containing PCR buffer, 0.2 mM deoxynucleotide triphosphates, 0.5 mM MgCl2, 1.25 U of Platinum® Taq Polymerase (Invitrogen), and 200 ng of template DNA. The final primer concentrations were 0.22 µM (for GSTP1*3, GSTT1, NAT2*6, and NAT2*7), 0.45 µM (for CYP1A2*1F and NAT2*5), and 0.16 µM (for GSTP1*2). PCR was conducted as follows: denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. For the duplex PCR, the final primer concentration was 0.1 µM, and PCR was performed with denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 59 °C for 60 s, and 72 °C for 90 s; and a final extension at 72 °C for 5 min (Fig. 1). In later experiments, the reaction volumes were reduced to 10 µL, and all fragments are amplified from 80 ng of DNA as starting material. After PCR amplification, the products were pooled (5 µL of the sevenplex and 4 µL of the duplex PCR product) and incubated (37 °C for 45 min) with 4 µL of ExoSAP-IT (Amersham) to digest contaminating deoxyribonucleotide triphosphates and primers. Enzymes were deactivated at 75 °C (15 min).

The primers for single base extension (SBE) were designed to bind immediately adjacent 5′ to the SNP. During thermal cycling, the primers are extended at their 3′ end with a single deoxyribonucleoside triphosphate labeled with a distinct fluorophore, revealing the genotype of the SNP. For GSTT1 and GSTM1 (deletions), the extension primers were designed to hybridize in the middle of the amplified fragment. All primers were designed to anneal to the antisense strand, except for the

References


